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Ribosomal ITS diversity among the European species of the genus *Hydnum* (Hydnaceae)

by

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Abstract

Grebenc, T., Martín, M.P. & Kraigher, H. 2009. Ribosomal ITS diversity in the European species of the genus Hydnum (Hydnaceae). *Anales Jard. Bot. Madrid* 66S1: 121-132.

Several morphological species of the genus *Hydnum* L. are known to occur in Europe, but little molecular evidence exists to confirm the exact number and delimitation of the species. The present study seeks to investigate the genus *Hydnum* through sequence analysis of the nuclear ribosomal ITS regions and through morphological studies. The DNA sequences phylogenetic analysis revealed high diversity among the ITS region sequences in *H. repandum* (two clades) and *H. rufescens* (six clades) while the specimens of *H. albidum, H. umbilicatum* and *H. ellipsosporum* formed one and clearly separated clade per morphological species. Phylogenetic distances among the recognised species and the obtained morphologically unsupported clades are comparable and support the idea of several new, yet undescribed species. The intraspecific variability in the sequence data among phylogenetic species is generally low. Detailed morphological analysis of putative informative morphological characteristics could not support any of the observed non-monophyletic DNA-sequences clades within *H. repandum* or *H. rufescens*, and the proper use of names is not yet clear. Similar intraspecific variation has also been observed in many other ectomycorrhizal genera and could be explained by intensive speciation within variable groups under the influence of various factors (niche effect, ectomycorrhizal partner selection).

Keywords: *Hydnum repandum*, *H. rufescens*, DNA-sequences phylogenetic relationships, morphological traits, nrDNA, intraspecific variability.

Resumen

Grebenc, T., Martín, M.P. & Kraigher, H. 2009. Diversidad de las secuencias ITS del ADN ribosómico nuclear en las especies del género Hydnum (Hydnaceae) en Europa. *Anales Jard. Bot. Madrid* 66S1: 121-132 (en inglés).

En Europa, sobre la base de la morfología se han identificado distintas especies en el género *Hydnum* L.; sin embargo, no se tenían datos moleculares para confirmar el número exacto de táxones y las relaciones entre los mismos. Este trabajo se basa en los análisis filogenéticos de las secuencias ITS del nrDNA, que se comparan con los estudios morfológicos y los análisis estadísticos. Los análisis filogenéticos revelan una alta diversidad en las secuencias de las regions ITS en *H. repandum* (dos clados) y en *H. rufescens* (seis clados), mientras que las muestras de *H. albidum, H. umbilicatum* e *H. ellipsosporum* se agrupan en clados únicos, que coinciden con especies tradicionales basadas en caracteres morfológicos. Los análisis morfológicos y filogenéticos son similares y apoyan la idea de que en este género existen todavía un número de especies no descritas. En las posibles especies filogenéticas, la variabilidad intraespecífica de las secuencias es baja. Por otro lado, el resultado del detallado análisis morfológico no apoya ninguno de los clados de *H. repandum* o *H. rufescens*, por lo que todavía no queda claro el táxon al que designan estos nombres. Una variabilidad intraespecífica similar se ha observado en otros géneros de hongos ectomicorrícicos y podría explicarse por especiación intensiva bajo la influencia de diversos factores (efecto de nicho, selección del hospedante ectomicorrícico).

Palabras clave: *Hydnum repandum*, *H. rufescens*, relaciones filogenéticas, caracteres morfológicos, ADN ribosómico nuclear, variabilidad intraespecífica.

Introduction

Members of the family Hydnaceae Chevallier (1826) are primarily identified by the presence of positive geotropic spines, ranging from small granular warts to clear individual spines (Ainsworth & al., 1973). Recent literature cites six valid genera in the family. To our knowledge, five of them were never included

in any molecular analyses: *Corallofungus* Kobayasi, *Dentinum* Gray, *Gloeomucro* R.H. Petersen, *Nigrohydnum* Ryvarden, and *Phaeoradulum* Pat. (Kirk & al. 2001); while *Hydnum* as the type genus, was the only genus represented in phylogenetic studies. At higher taxonomic rank *Hydnum* was placed in *Cantharellales* first by Kreisel (1969) and later confirmed with molecular data by Pine & al. (1999) and subsequent papers.

In European taxonomic reviews and determination books the following species and varieties/forms have been mentioned in the genus: *Hydnum albidum* Peck, *H. repandum* L.:Fr. and *H. rufescens* Pers. (Maas Geesteranus, 1975; Jülich, 1984; Courtecuisse & Duhem, 1995), *H. repandum* var. *rufescens* (Fr.) Barla and *H. repandum* f. *rufescens* (Pers.) Nikol. (synonym of *H. rufescens* Pers.) (Marchand, 1973; Cetto, 1976; Gerhardt, 1997). In Slovenia two more taxa have been cited: *H. umbilicatum* Peck (Petkovšek & Vrščaj, 1998) [the species generally known from North America (Hall & Stuntz, 1971) and Asia (Maas Geesteranus, 1971)] and *H. repandum* f. *amarum* Vrščaj (Stropnik & al., 1988); however, the second taxon was never published with a comprehensive description, thus according to Art. 36.1, CABI Bioscience Databases (Kirk & al., 2003) the name *H. repandum* f. *amarum* Vrščaj is treated as *nom. inval.*

Hydnum rufescens and *H. repandum* are distributed over an exceptionally wide area and are even recognised in the Far East (Asia) although several synonyms from different areas and for local populations were published and many local names were conspecific with European species (Maas Geesteranus, 1971). *Hydnum elatum* Massee and two more unnamed *Hydnum* species were recognised for Asia and Australia in addition to *H. umbilicatum* (Maas Geesteranus, 1971). The latter was described in North America by Peck (1902). Despite it was commonly found on several continents (Hall & Stuntz, 1971) its presence in Europe was only confirmed for Finland (Huhtinen & Ruotsalainen, 2006).

Clear delimitation of species cited in Europe is not always easy. Morphological characters can vary with the developmental stage of pileus and environmental conditions during the growth period (Hall & Stuntz, 1971; Maas Geesteranus, 1975). Spore size and shape can well separate *H. albidum* and *H. ellipsosporum* from the others (Ostrow & Beenken, 2004) while taxonomical position of *H. rufescens* within the genus is confusing, not only after classical identification but also after molecular data have become available. Molecular identification of *H. repandum* and *H. rufescens* ectomycorrhizae on Norway spruce showed distinct restriction patterns of amplified ITS region in genomic rDNA. Additionaly the variability of the restriction pattern within *H. rufescens* was observed after digestion of the amplified PCR product with *Hinf*I endonuclease. The observed additional differences indicate possible variability of collections from different sites (Agerer & al., 1996). Ostrow & Beenken (2004) found a good correlation for selected morphological and molecular characters for four European species with only few samples sequenced for each species. They reported no sequence diversity within *H. rufescens,* although only for *H. repandum* and *H. ellipsosporum* was the absence of any such intraspecific variability clearly stated.

Comparison of rDNA ITS sequences is a valuable tool in phylogenetic studies, and to provide more accurate species delimitation (Taylor & al., 2000). Currently there is a poor overlap between morphological and molecular species concept based on the variability of the rDNA ITS sequences in studied *Hydnum* collections. To support the results obtained at the molecular level, selected morphological characters indicative of taxonomic affiliation in the genus *Hydnum* were measured and correlated to the clades retrieved in the DNA-sequences phylogenetic analyses. Multivariate statistics were employed for these analyses.

Materials and methods

DNA analyses were undertaken in the laboratories in Slovenia (SFI) and in Spain (RJB). The different protocols were standardized at both sites, such that the final results obtained from the same sample were equal. Thin layer cromatography (TLC) analysis was carried out in RJB in Spain.

Fungal material

Specimens from the genus *Hydnum* included in the study (Table 1) were either collected from various localities in the years 1999-2002 and stored in the herbarium at Slovenian Forestry Institute (LJU) or obtained from herbarium MA-Fungi (Madrid, Spain). We have tried to locate the type or representative material for European species in different institutional herbaria (UPS, MSB, and PC; Holmgren & al., 1998). However, according to the curators, the material either does not exist or was not possible to locate. Even though *H. umbilicatum* has been cited in Slovenia, no reference material was available from the area; the two collections included in the study were kindly sent by Lorelei L. Norvell from the Pacific Northwest Mycology Service.

Specimens with fully developed basidiomata and spores were used for examination of macro- and mi-

croscopic morphological characters. Fifty spores per basidiomata were measures to calculate an average and extreme values for length, width, and spore volume (Ostrow & Beenken, 2004) and to assess several other potential informative characters (cap colour, stem diameter and position, cap diameter, spine position, pigmented content, and potential ectomycorrhizal partners on the collection site). Nomenclature followed the morphological species concepts of Maas Geesteranus (1975) *(H. repandum* and *H. rufescens),* Focht (1996) and Ostrow & Beenken (2004) for *H. albidum,* and Harrison & Grund (1987) for *H. umbilicatum,* the only non-European species included in the study.

Molecular methods

DNA extraction: Twenty milligrams of the hymenium from fresh or dried material were used for the DNA extraction following standard protocols after Whiting & al. (1997) or using 2% CTAB (Rogers & Bendlich, 1985; Doyle & Doyle, 1990). From older herbarium material DNA was extracted by E.Z.N.A. Fungi DNA Miniprep Kit (Omega Biotek) as described in Martín & García-Figueres (1999). For both methods DNA was re-suspended in pre-warmed, sterile milli-Q water to the approximate final concentration 100 ng/ml.

PCR amplification: Primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White & al., 1990) or ITS4b (Gardes & Bruns, 1993) were used for PCR amplification of the ITS region, including 5.8 S rDNA. Amplification reactions were obtained using two methods: a) standard procedure described in White & al. (1990) in a total reaction volume of 40 µl with AmplyTaq polymerase (Perkin Elmer) and/or b) individual reactions in a final volume of 25 µl with Ready-To-Go PCR Beads (GE Healthcare Life Sciences) as mentioned in Winka & al. (1998). The PCR reactions were performed after Kraigher & al. (1995) in a PE 9700 DNA thermocycler with an annealing temperature 55°C. Negative controls, lacking fungal DNA, were run for each experiment to check for any contamination of the reagents. Amplified DNA was separated and analysed as described in Grebenc & al., 2000.

Sequencing and cloning: Prior to sequencing, the amplification products were cleaned using the E.Z.N.A. Clean kit. When only weak PCR products were obtained the products were cleaned from the gel using QIAquick Gel (QIAGEN Inc.), cloned with pGEM®-T Easy Vector Systems (Promega), and purified with QIAPrep Spin Mini prep. Three clones were selected for sequencing with vector specific primers T7 and SP6 (QIAGEN Inc.). Sequence Navigator Software (Applied Biosystems) was used to identify the consensus sequence from the two strands of each isolate. When the sequences obtained from the cloned products were identical, only one sequence was included in the alignment. The sequences were submitted to EMBL database with the accession numbers indicated in Table 1.

DNA-sequences phylogenetic analyses: Se-Al v2.0a11 software (http://tree.bio.ed.ac.uk/software/seal/) for multiple sequences was used to search for the best alignment of new sequences, and sequences from the genus *Hydnum* already deposited in GenBank or UNITE databases [*H. albidum* (ALB, AY817135); *H. albomagnum* (ALM, DQ218305); *H. ellipsosporum* (ELL, AY917139); *H. repandum* (REP1, AJ889978; REP2, AJ889949; REP3, AY817136; REP4, DQ218306; REP5 DQ367902; REP6, UDB000025; REP7, UDB000096; REP8, UDB001479); *H. rufescens* (RUF1, AY817137; RUF2, DQ658890; RUF3, AM087246; RUF4, UDB001465; RUF5, UDB002423); *H. umbilicatum* DQ367903].

Where ambiguities in the alignment occurred, the alignment chosen was the one generating the fewest potentially informative characters. Alignment gaps were marked "-", unresolved nucleotides and unknown sequences were indicated with "N". The alignment was analysed using the programmes PAUP *Version 4.0b10 for Macintosh (Swofford, 2002) and MrBAYES 3.0 (Huelsenbeck & Ronquist, 2001). As an outgroup for the DNA-sequences phylogenetic analysis, we have selected *Sistotrema muscicola* (Pers.) S. Lundell isolate KHL11721 (AJ606040), and *Sistotrema alboluteum* (Bourdot & Galzin) Bondartsev & Singer isolate TAA180259 (AJ606042), both ectomycorrhizal and closely related to the genus *Hydnum* (Moncalvo & al., 2006; De Marino & al., 2008).

A first maximum parsimony analysis (MP) was inferred using the heuristic search option of the 100 most parsimonious trees in PAUP*4.0b10. Gaps were treated as missing data. Branch lengths equal to zero were collapsed to polytomies. Nonparametric bootstrap support (Felsenstein, 1985) for each clade was tested based on 10 000 replicates, using the fast-step option. The consistency index, CI (Kluge & Farris, 1969), retention index, RI (Farris, 1989), and rescaled consistency index, RC (Farris, 1989) were obtained.

A second analysis was carried out using a Bayesian approach (Huelsenbeck & al., 2000; Larget & Simon, 1999). Posterior probabilities were approximated by sampling trees using a Markov Chain Monte Carlo (MCMC) method. The posterior probabilities of each branch were calculated by frequency of trees that were visited during the course of the MCMC analysis.

Table 1. *Hydnum*. Collections included in the sequence analyses. Species were determined after morphological characteristics. Taxon names, location, potential host (s), herbarium voucher, DNA isolation code and GenBank accession numbers (Acc. Num.) are given.

Taxon name	Location	Potential host(s)	Herbarium voucher	Code[1]	Acc. Num.[2]
H. albidum	Divaški gabrk, Divača, Slovenia	*Pinus nigra*	LJU GIS 1341	ALBHYD1*	AJ534974
H. albidum	Merindad de Valdivieso, Burgos, Spain	*Pinus pinaster*	MA-Fungi 40149	ALBHYD2	AJ534975
H. repandum f. *amarum*	Velike Lašče, Slovenia	*Picea abies, Fagus sylvatica*	LJU GIS 1337	REPHYD1	AJ547871
H. repandum	Kalič, Slovenia	*Fagus sylvatica, Abies alba, Acer pseudoplatanus*	LJU GIS 1342	REPHYD3	AJ547874
H. repandum	Rajhenavski Rog virgin forest, Žaga-Rog, Slovenia	*Abies alba, Fagus sylvatica*	LJU GIS 1345	REPHYD4	AJ547876
H. repandum	Rajhenavski Rog, Žaga-Rog, Slovenia	*Abies alba, Fagus sylvatica*	LJU GIS 1344	REPHYD5	AJ547875
H. repandum	Gore, Idrija, Slovenia	*Picea abies, Fagus sylvatica*	LJU GIS 1322	REPHYD6	AJ547877
H. repandum	Čekovik, Idrija, Slovenia	*Picea abies, Fagus sylvatica*	LJU GIS 1321	REPHYD7	AJ547878
H. repandum	Vače, Slovenia	*Pinus sylvestris*	LJU GIS 1326	REPHYD8	AJ547881
H. repandum	Pusti Javor, Šmartno pri Litiji, Slovenia	*Picea abies, Fagus sylvatica*	LJU GIS 1325	REPHYD9	AJ547883
H. repandum	Andorra	*Pinus* sp., *Corylus avellana*	MA-Fungi 47727	REPHYD10	AJ547888
H. repandum	Velike Lašče, Slovenia	*Picea abies, Fagus sylvatica, Corylus avellana*	LJU GIS 1334	REPHYD11	AJ547886
H. repandum	Castañar de Ibor, Cáceres, Spain	*Castanea sativa*	MA-Fungi 3457	REPHYD12A	AJ547879*
				REPHYD12C	AJ783968*
H. repandum	El Serrat, Andorra	*Corylus avellana, Buxus sempervirens*	MA-Fungi 47726	REPHYD13	AJ547887
H. ellipsosporum	Grajski Boršt, Slovenia	mainly *Fagus sylvatica*	LJU GIS 1327	RUFHYD1	AJ535304
H. rufescens	Pringl, Idrija, Slovenia	*Picea abies, Fagus sylvatica, Acer pseudoplatanus*	LJU GIS 1320	RUFHYD2	AJ535301
H. rufescens	Pusti Javor, Šmartno pri Litiji, Slovenia	*Picea abies, Fagus sylvatica*	LJU GIS 1329	RUFHYD3*	AJ535303
H. cf. *rufescens*	Pusti Javor, Slovenia	*Picea abies, Fagus sylvatica*	LJU GIS 1328	RUFHYD4	AJ535302
H. cf. *rufescens*	Ilova gora, Slovenia	*Picea abies*	LJU GIS 1324	RUFHYD5	AJ547869
H. cf. *rufescens*	Polica, Grosuplje, Slovenia	*Picea abies, Fagus sylvatica*	LJU GIS 1340	RUFHYD6*	AJ547884
H. ellipsosporum	Canebela, Orense, Spain	*Fagus sylvatica, Castanea sativa*	MA-Fungi 47725	RUFHYD8	AJ547882
H. rufescens	Close to Velike Lašče, Slovenia	*Picea abies, Pinus sylvestris, Fagus sylvatica, Quercus* sp.	LJU GIS 1333	RUFHYD9	AJ535305
H. rufescens	Pringl, Idrija, Slovenia	*Picea abies, Fagus sylvatica, Acer pseudoplatanus*	LJU GIS 1323	RUFHYD10	AJ547866
H. rufescens	El Serrat, Andorra	*Pinus* sp., *Corylus avellana*	MA-Fungi 47728	RUFHYD11	AJ547889
H. rufescens	Mežjanca, Radovna, Slovenia	*Picea abies*	LJU GIS 1331	RUFHYD12	AJ783969
H. rufescens	Ilovce, Idrija, Slovenia	*Picea abies, Fagus sylvatica*	LJU GIS 1330	RUFHYD14*	AJ547872
H. rufescens	Nova vas, Slovenia	*Picea abies*	LJU GIS 1339	RUFHYD15	AJ547867
H. rufescens	Rajhenavski Rog virgin forest, Žaga-Rog, Slovenia	*Abies alba, Fagus sylvatica*	LJU GIS 1332	RUFHYD16*	AJ547868
H. rufescens	Velike Lašče, Slovenia	*Picea abies, Fagus sylvatica, Corylus avellana*	LJU GIS 1336	RUFHYD17	AJ547885
H. umbilicatum	Green Peak, Benton County, USA	*Pseudotsuga menziesii, Acer circinatum*	PNW-MS g2010502h1-09	UMBHYD1	AJ534972
H. umbilicatum	Green Peak, Benton County, USA	*Pseudotsuga menziesii, Acer circinatum*	PNW-MS g2011128m2-09	UMBHYD2	AJ534973

[1] Collections included in the TLC analyses are marked with * next to the code.
[2] Sequences obtained after cloning are marked with * next to the Accession Number.

The analysis was performed assuming the general time reverse model (Rodriguez & al., 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six categories (GTG+I+G). No molecular clock was assumed. A run with 10 000 000 generations starting with a random tree and employing 12 simultaneous chains was executed. Every 100th tree was saved into a file of total of 100 000 trees. We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 (http://evolve.zoo.ac.uk/software.html?i=tracer) and determined that stationarity was achieved when the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist, 2001). The initial 1000 trees were discarded as burning before stationarity was reached. Using the "sumt" command of MrBAYES, majority-rule consensus trees were calculated from 19 000 trees sampled after reaching likelihood convergence to calculate the posterior probabilities of the tree nodes. DNA-sequences phylogenetic trees were drawn in TREEVIEW (Page, 1996).

TLC analysis

Pigment composition was analysed using TLC for selected collections only (Table 1) based on their position in DNA-sequences phylogenetic tree so at least one sample from each clade was included. The acetone extractions from herbarium material were developed (TLC) in the solvent system benzene: ethyl formate: formic acid (10:5:3) in standard Brinkman tanks on 20 × 20 cm Merck pre-coated Silica Gel F254 plates (Gill & Steglich, 1987). Norbadion, badion A, badion B, boviquinon, involutin, gyroporin, telephoric acid, atromentin, variegatic rubin, variegatic acid, xerocomic acid, L-tyrosin, L-DOPA, and ergosterol were used as standard marker pigments. Instead of pure pistillarin we have used a *Ramaria cedretorum* (Maire) Malençon (MA-Fungi 48074), specimen with positive reaction for pistillarin (Daniëls, 2002). After removal from the tanks, the plates were air dried and examined under visible light and under UV-lamp (245nm and 366nm) following Martín & Sánchez-Cuixart (1996).

Results

A total of 32 new ITS nrDNA sequences were generated. The sequences were aligned with 17 ITS nrDNA sequences available from Genbank and UNITE to produce a matrix of 675 unambiguously aligned nucleotide position characters of which 464 were constant, 71 variables are parsimony uninformative, and 140 parsimony informative. The alignment with 52 sequences is available at TreeBASE (http://www.treebase.org/, as SN2182).

Maximum parsimony analysis (MP) under heuristic search gave 12 most parsimonious trees with a length of 345 steps, CI = 0.6870, RI = 0.8636, and RC = 0.5933. Figure 1a shows the strict consensus tree where the bootstrap support values (bs) above 50% are indicated on the branches and DNA-sequences phylogenetic tree generated by the Bayesian analysis (Fig. 1b). The likelihood parameters of the MP analysis had the following average values (± one standard deviation): likelihood = – base frequencies $\pi(A) = 0.2343$ (± 0.00018), $\pi(C) = 0.193$ (± 0.00017), $\pi(G) = 0.224$ (± 0.00018), $\pi(T) = 0.34$ (± 0.00018), rate matrix r(AC) = 2.652 (± 0.099), r(AG) = 19.289 (± 0.088), r(AT) = 1.729 (± 0.099), r(CG) = 1.098 (± 0.001), r(CT) = 12.285 (± 0.089), r(GT) = 1.0 (± 0.0), gamma shape parameter alpha = 0.655 (± 0.0026) and the proportion of invariable site p(invar) = 0.255 (± 0.0029). From the Bayesian tree, where the posterior probabilities (pp) were indicated on the branches, only the major, well-supported clades will be discussed in the following.

Both analysis methods applied yielded an identical distribution of terminal clades although the distribution of deeper clades varies slightly. *Hydnum albidum* forms a single clear clade (AL1, Fig. 1). *H. repandum* formed two closely related clades (RE1 and RE2, Fig. 1) separated by *H. rufescens* clade (RU1, Fig. 1). Analysed sporocarps from RU1 show intermixed morphological characteristics between *H. rufescens* and *H. repandum* (Table 2). The *H. rufescens* samples were distributed among six more clades (RU1-6, Figs. 1), intemixed with the two sister species *H. ellipsosporum* and *H. umbilicatum*.

In order to cast additional light on the molecular differences among observed *H. rufescens* and *H. repandum* clades, we applied a TLC chromatography of the pigments in the basidiomata of one collection from each clade (see Table 1). Under visual light no spots of pigment were recognised after the chromatography. Under the illumination with UV (wave length 302 nm) spots appeared at Rf: 87.5 (close to ergosterol or L-DOPA), Rf: 73.5, and a longer spot with Rf: 65-59. All samples gave the same pigment composition, though only the intensity of coloration of sporocarps was represented.

Several other potential taxonomic informative characteristics were assessed for samples within each of the clades for possible separation of observed terminal clades in DNA-sequences phylogenetic trees at the morphological level (Table 2). Multiple range tests for

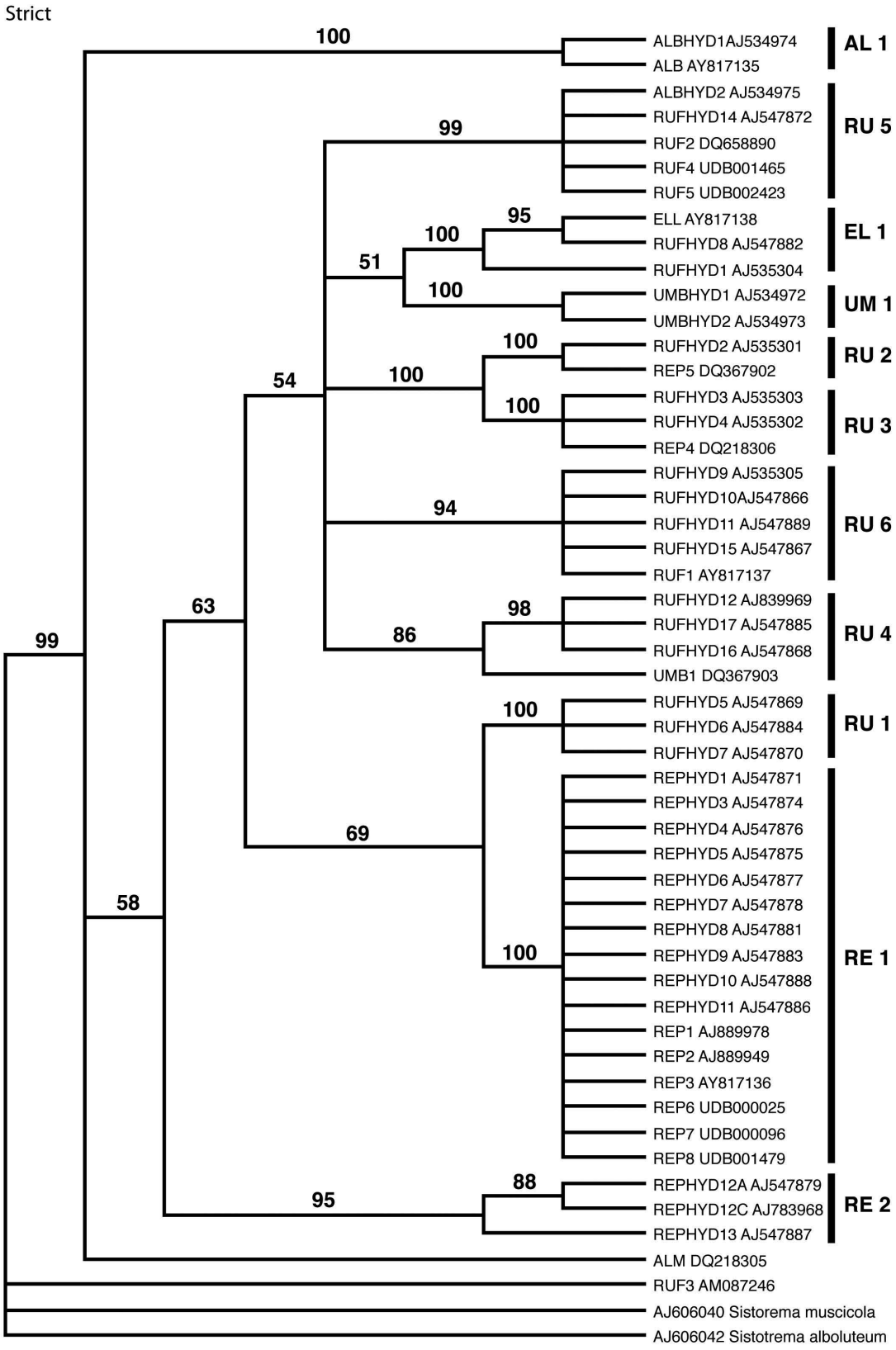

Fig. 1a. DNA-sequences phylogenetic tree for the *Hydnum* specimens under study. Strict consensus tree with bootstrap values for heuristic search of the 100 most parsimonious trees. DNA-sequences phylogeny clades/morphological species: RU, *H. rufescens*; RE, *H. repandum*; UM, *H. umbilicatum*; EL, *H. ellipsosporum*; AL, *H. albidum* and ALM, *H. albomagnum*. OTUs names using codes in Tables 1 and 2, followed by the Accession Number from the GenBank or UNITE databases.

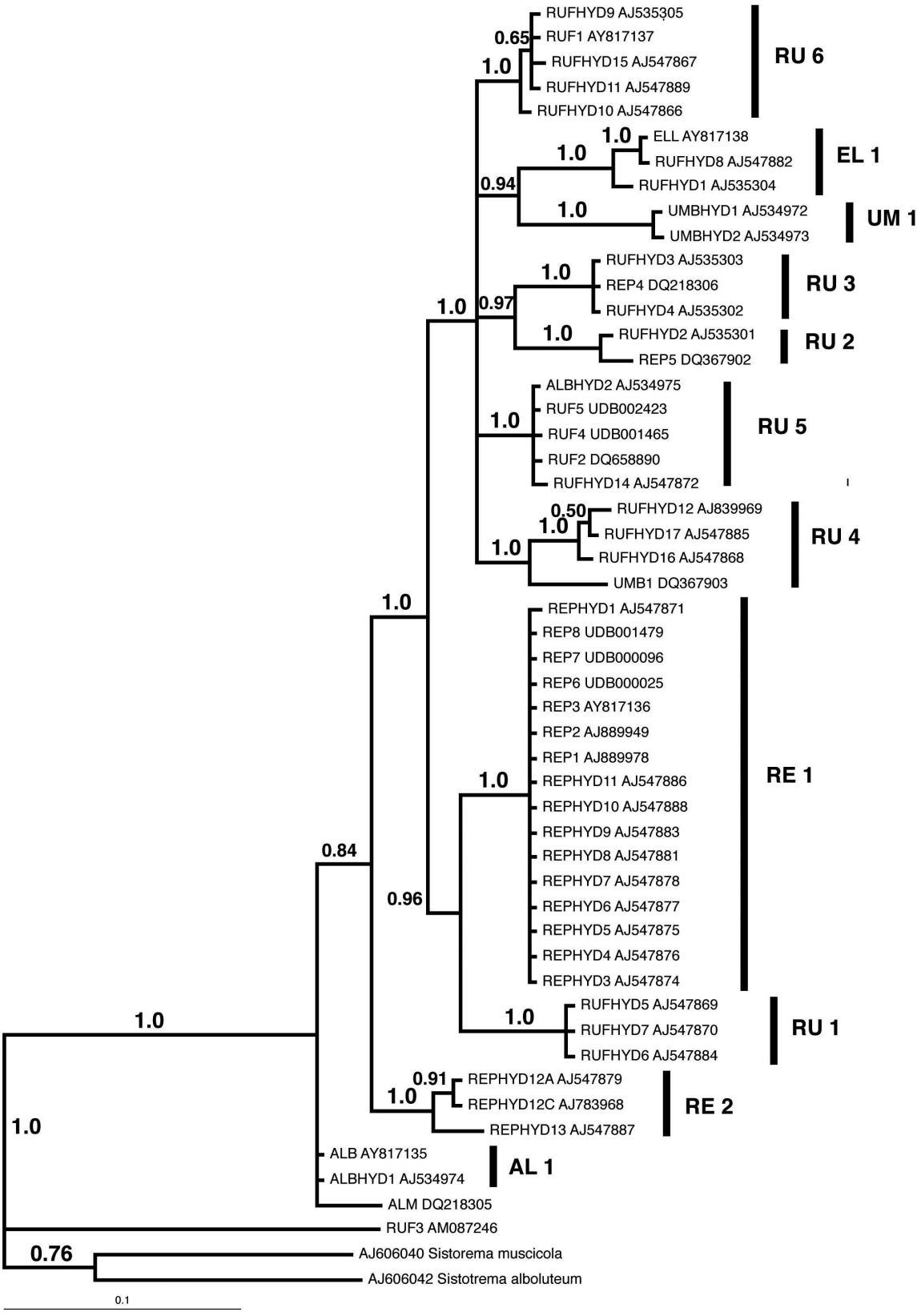

Fig. 1b. DNA-sequences Bayesian phylogenetic tree for the *Hydnum* specimens under study, with posterior probabilities. DNA-sequences phylogeny clades/morphological species: RU , *H. rufescens*; RE, *H. repandum*; UM, *H. umbilicatum*; EL, *H. ellipsosporum*; AL, *H. albidum* and ALM, *H. albomagnum.* OTUs names using codes in Tables 1 and 2, followed by the Accession Number from the GenBank or UNITE databases.

spore size and volume clearly separated *H. albidum, H. umbilicatum*, and *H. ellipsosporum* from *H. repandum* and *H. rufescens* clades, while clades within the latter two morphological species could not be clearly separated, except based on cap colour. We have observed no significant statistical difference between characters within either *H. repandum* or *H. rufescens* clades.

Discussion

The last comprehensive revision of the family Hydnaceae was published by Maas Geesteranus (1975) based on morphological characteristics of basidiomata and spores. Unequivocally defined taxa are a prerequisite for a comprehensive ecological, physiological, or molecular analysis of a taxon. The aim of this study was: to support the established taxa as recognised after the morphological concept of the species within the European *Hydnum* species, employing molecular tools, and to clarify the observed molecular differences in the previous study (Agerer & al., 1996). The value of the presented results would be greater if type material existed and was available at least for *H. repandum* and *H. rufescens*.

The basidiomata included in the study were primarily identified based on morphological characteristics. Spore size and shape can be a good criterion to separate *Hydnum albidum*, *H. ellipsosporum* (Ostrow & Beenken, 2004), and *H. umbilicatum* (Hall & Stuntz, 1971) from the rest of *Hydnum* species from Europe. Huhtinen & Ruotsalainen (2006) examined the material from Finland and were able to separate all together three taxa within *"H. rufescens"* specimen: *H. ellipsosporum, H. umbilicatum* and *H. rufescens* s. str. with at least two separate populations in the latter species based on spore shape. However, the data from several identification books/keys (Maas Geesteranus, 1975; Jülich, 1984; Courtecuisse & Duham, 1995; Ostrow & Beenken, 2004), as well as the results of the present study indicate that spores of *H. rufescens* and *H. repandum* do not differ significantly in size or shape. The basidiome size, position of the stipe, distribution of the spines, and shape and colour of the basidiome were relatively reliable morphological criteria to distinguish these two morphological species without the need to employ molecular tools, yet our results indicate much higher diversity within these two species. An umbilicate pileus, otherwise typical for *H. umbilicatum* (Hall & Stuntz, 1971), was observed for some collections of *H. rufescens* from Europe but observed morphological characteristics do not indicate the presence of *H. umbilicatum* in Europe since the spore size is within the range of *H. rufescens* and the umbilicate pilei were distributed among different DNA-sequences phylogenetic clades obtained (data not shown). The exception is a collection from Finland which was microscopically identified as *H. umbilicatum* but not anaysed at DNA level (Huhtinen & Ruotsalainen, 2006). In specimens of *H. umbilicatum* collected in North America (Harrison & Grund, 1987) or Asia (Maas Geesteranus, 1971), the spores are larger than for other species and form a unique clade within the DNA-sequences phylogenetic tree, similary distant from several *H. rufescens* clades and *H. ellipsosporum*. Solely using morphological criteria a misidentification of *H. rufescens* with *H. umbilicatum* would only be possible for samples with extremely large spores which might explain the use of the name "umbilicatum" in some European literature.

Hydnum ellipsosporum was first described only recently, based on many collections from Germany (Ostrow & Beenken, 2004). The distribution of this species seems to be broader since two collections analysed in our study were from Spain (RUFHYD8) and Slovenia (RUFHYD1), at both locations collected from sites dominated by broadleaved trees, which is not common for other *Hydnum* collections. The species was also confirmed from various locations in Finland. After the DNA-sequences phylogenetic analyses, we confirmed Ostrow & Beenken's well supported molecular and morphological separation of the species from other European species of *Hydnum*.

Based on DNA-sequences, the phylogenetic separation within *H. repandum* and *H. rufescens* indicates higher DNA-sequences variability than that observed solely at the morphological level. Within DNA-sequences phylogenetic trees based on ITS1, 5.8S and ITS2 sequences nrDNA, *H. repandum* specimens formed two non-monophyletic but well supported clades (RE1 and RE2) after both maximum parsimony and Bayesian analysis. The only morphological criterion to separate these two clades is the size of pileus, which appeared smaller for collections distributed in clade RE2. Additionally, clade RU1 (samples identified as *H. rufescens*, but with mixed morphological characteristics of *H. rufescens* and *H. repandum*; see Table 2) appeared as neighbour clade. In this case, despite being uncommon in fungi (Spiers and Hopcroft, 1994), hybridisation cannot be excluded. The resolution obtained from the ITS region was not enough to distinguish *H. repandum* and *H. repandum* f. *amarum* (HYDREP1), a locally recognised form reported to be non-edible due to its bitter taste at all developmental stages of the fruitbody (Stropnik & al., 1988; Petkovšek & Vrščaj, 1998). A somewhat bitter taste of some basidiomata of *H. repandum* has been

Table 2. Summary data (values are summarized for all measurements and collections available, averaged and rounded) and statistical analysis (Multiple Range Test, $p<0.05$; A-D – similarity groups) for potential informative morphological characters assessed on all available collections distributed among each DNA-sequences phylogenetic clade obtained.

Clade	Spore length (µm)	Spore width (µm)	Spore volume (µm³)	Cap colour	Stem position	Stem diameter (mm)	Cap diameter (mm)	Spines	Number of potential ECM partners on site
AL	4-6 (4,8)^{A}	2,5-4,5 (3,4)^{A}	13,8-70,5 (34,9)^{A}	Bright ochre^{C}	Central	6-13 (8,8)^{BCD}	40-65 (54)^{CD}	Decurrent	1
UM	7,5-10 (8,8)^{D}	6-8,5 (7,2)^{C}	159,0-425,6 (266,8)^{D}	Bright yellow^{A}	Central	3-6 (3,5)^{AB}	15-35 (21)^{AB}	Free	2
EL	6-10 (8,8)^{D}	6-10 (7,3)^{C}	163,4-485,4 (284,1)^{D}	Intensive yellow-orange^{BC}	Central	4-8 (6,5)^{ABCD}	21-55 (36)^{ABCD}	Free	2
RE1	5-8 (7,4)^{C}	5,5-9 (6,5)^{C}	82,8-340,3 (196,4)^{C}	Bright yellow^{A}	Excentric	5-25 (9,5)^{D}	25-95 (46)^{D}	Decurrent	2
RE2	6-9,5 (7,6)^{C}	5-8 (6,5)^{C}	116,5-292,1 (194,6)^{BC}	Bright yellow^{A}	Excentric	3-8 (4,5)^{ABC}	21-50 (32)^{ABC}	Decurrent	1
RU1	7-8 (7,0)^{B}	5-7 (6,3)^{BC}	92,0-226,2 (164,7)^{BC}	Bright-intensive yellow^{B}	Central, occasional excentric	3-7 (5)^{BCD}	20-100 (89)^{D}	Free	1
RU2	5-8 (6,9)^{BC}	5-7,5 (6,1)^{BC}	110,7-2589 (157,7)^{BC}	Intensive yellow-orange^{B}	Central	3-5 (4)^{ABC}	10-35 (23)^{A}	Free	1
RU3	6-9 (7,1)^{BC}	5-8 (6,5)^{C}	92,1-316,0 (183,7)^{BC}	Bright yellow^{A}	Central	5-9 (8)^{CD}	20-53 (41)^{BCD}	Free	2
RU4	5,5-9 (7,5)^{C}	5-8 (6,6)^{C}	87,4-325,4 (193,4)^{BC}	Intensive yellow-orange^{B}	Central	3-5 (4)^{A}	18-32 (22)^{A}	Free	1-3
RU5	5,5-9,5 (7,9)^{C}	5-9 (6,8)^{B}	82,8-394,6 (219,3)^{B}	Intensive yellow-orange^{B}	Central	4-8 (5,5)^{ABC}	20-32 (23)^{AB}	Free	2
RU6	5,5-9,5 (7,6)^{C}	5-8 (6,7)^{C}	92,0-340,3 (203,5)^{C}	Intensive yellow-orange^{B}	Central, occasional excentric	3-8 (5,5)^{ABC}	18-46 (31)^{AB}	Free	1-4

reported by other authors for collections from Asia. A similar taste has also occasionally been noticed in Europe by Jaccottet in 1948 (cit. in Maas Geesteranus, 1971) but it was never proposed to form a separate taxonomical unit. No data is available for constant and uniform occurrence of basidiomata with a bitter taste. This character may be due to the influence of ecological conditions of the site.

Hydnum rufescens, the only species within the genus with previously confirmed intraspecific variability (Agerer & al., 1996), appeared to be the most variable. The specimens were found to fall into six well supported but non-monophyletic clades after DNA-sequences phylogenetic analysis. The DNA-sequences phylogenetic tree distances between each of the neighbouring *H. rufescens* clades and other sister clades (e.g. well established species *H. umbilicatum* and *H. ellipsosporum)* indicate that each of the six *H. rufescens* clades can be recognised and treated as separate species. The morphological information assessed did not correlate well with the molecular results. Rough statistical analysis can only separate group RU3, with generally larger sporocarps but no significant difference in spore size, and no other assessed characteristics could be found for any of the other clades.

The main evolution force for ribosomal region is a concerted evolution which should lead to homogenisation of individual repeats and produce a uniform sequence in all repeats of a given phylogenetic species (Vogler & DeSalle, 1994). Differences in sequences within one morphological species, as observed in *H. repandum* and *H. rufescens,* may indicate the presence of more than one phylogenetic species (cryptic species) or variation within the species on a molecular level which cannot be explained by the concerted evolution theory. Similar variation and presence of more phylogenetic species was earlier observed and proposed within *Rhizopogon roseolus* (Martín & al., 2000), *Tricholoma flavovirens* (Pers.) S. Lundell. (Horton, 2002), *Tuber rufum* (Iotti & al., 2007; Grebenc & al., in prep), and in other ectomycorrhizal genera (*Leccinum, Lactarius, Inocybe, Tricholoma,* and *Russula)* (Kåren & al., 1997; Horton, 2002). This could be the case in *Hydnum rufescens* and *H. repandum* as well. Relatively high abundance of such variability seriously challenges the morphological species concept for these taxa. Several studies demonstrated that DNA-sequences based phylogenetic species recognition and concept were advantageous in mycology and seem likely to become popular among mycologists (Taylor & al., 2000). It could well be applied in the case of *H. rufescens, H. repandum,* yet raising the question about the use of current names for several phylogenetic species.

Despite relatively high genetic distance in the DNA-sequences phylogenetic tree, the geneflow between clades cannot be excluded, but the presence of putative heteroduplex in rDNA was rejected using DGGE analysis of ITS region (Grebenc & al., 2006).

Based on the high molecular diversity of presumably homogeneous rDNA region in *H. rufescens* and *H. repandum* these two species may be in a process of intensive speciation, not correlated to the geographical distances between the different clades obtained. There are several possible triggers which could lead to a possible diversification at the molecular level in *H. rufescens* and *H. repandum*. Harrington & Rizzo (1999) reported a high importance of niche in determining the development and maintenance of fungal species which is not necessarily correlated to geographical distances. *H. rufescens* and *H. repandum* are common species in Europe growing next to one or more different ectomycorrhizal partners (Table 1) which could lead to a possible diversification at the molecular level, as observed in the *H. ellipsosporum* samples analysed. Other ecological variables not quantified in the present study, for example soil and other environmental parameters, possible niche specialist character of the species (Giraud & al., 2008), or ectomycorrhiza characteristics and partners, mating types, etc., should be evaluated to explain the variability at the molecular level.

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